

5 *XhoI/SalI* (filled in with Klenow) linearized pRS416 CYC1  
(Mumberg, *et al.*, *Gene* **156**:119-122 (1995)).

MB1644 and MB2478 are *URA3*-based *S. cerevisiae*  
expression plasmids that contain the wild-type *lovE* gene.  
They are both derivatives of MB1199. MB1199 was created  
10 by using primers MO841 (SEQ ID NO:11) and MO842 (SEQ ID  
NO:12) to amplify the *lovE* ORF from *A. terreus* cDNA.  
Gateway (Invitrogen™, Carlsbad, CA) Cloning Technology (US  
Patent 5,888,732) was used to clone the *lovE* PCR fragment  
into the gateway entry vector pDONR206 (Invitrogen™,  
15 Carlsbad, CA) to create MB1199. Similarly, Gateway  
Cloning Technology was used to transfer the *lovE* ORF from  
MB1199 into MB968 to create MB2478 and into MB969 (U.S.  
Serial No. 60/198,335, filed April 18, 2000) to create  
MB1644.

20 MB2848 is a derivative of MB968 that contains a *lovE*-  
*AT274* chimera. The *lovE* portion of MB2848 was derived by  
using oligos MO841 (SEQ ID NO:11) and MO2278 (SEQ ID  
NO:13) to PCR amplify the *lovE* DNA binding domain from *A.*  
*terreus* cDNA. A second round of PCR was performed with  
25 primers MO343 (SEQ ID NO:14) and MO2278 to add appropriate  
Gateway Cloning Technology compatible sequences. The  
*At274* portion of MB2848 can be derived by using primers  
MO2273 (SEQ ID NO:15) and MO2274 (SEQ ID NO:16) to PCR  
amplify the carboxy-terminal domain of *At274* from *A.*  
30 *terreus* cDNA. A second round of PCR was performed with  
primers MO344 (SEQ ID NO:17) and MO2273 to add appropriate  
Gateway Cloning Technology compatible sequences. The *lovE*  
and *At274* PCR products were cut with *BamHI* and purified  
over a QIAquick PCR purification kit (Qiagen, Valencia,  
35 CA) according to manufacturer's instructions. Finally,  
the products were mixed 3-4 hours in a standard ligation  
reaction and used in Gateway entry and destination  
reactions to create MB2848.

Gateway cloning technology was used to clone the *lovE*  
40 variants of interest into plasmid MB1419 which is a  
filamentous fungal expression vector. The MB1419 fungal  
selection marker is the *A. nidulans* *GPD* promoter  
controlling the *ble* gene from *S. hindustanus*. The

5 transgene is controlled by the *A. nidulans* PGK promoter.  
*A. terreus* strain MF117 is a derivative of *A. terreus*  
strain ATCC 20542.

**Example 2: PCR Mutagenesis of the *lovE* DNA Binding Domain**

10 The zinc finger DNA binding domain of *lovE* is encoded by  
nucleotides 100-201 (SEQ ID NO:92). Oligos MO2624 (SEQ ID  
NO:18) and MO2654 (SEQ ID NO:19) were used to PCR amplify  
a *lovE* containing fragment from plasmid MB2478. The 1.7  
kb product contains nucleotides 212-1410 of *lovE* and ~500  
15 bp of flanking vector sequence. Two rounds of standard  
PCR (1.5 mM MgCl<sub>2</sub>) were performed with Amplitaq DNA  
polymerase (Applied Biosystems, Foster City, Ca) according  
to the manufacturer's instructions.

Plasmid MB2848 was cut with *KpnI*-*Bam*HI to release a 1.1  
20 kb fragment containing the *At274* portion of the *lovE*-*At274*  
chimeric open reading frame. The remaining 5.5 kb vector  
sequence retains the *lovE* DNA binding domain.

**Example 3: PCR Mutagenesis of the *lovE* Open Reading Frame**

25 *lovE* open reading frame insert was prepared according  
to the following procedure. Oligo pairs MO2680 (SEQ ID  
NO:20) /MO2686 (SEQ ID NO:21), MO2681 (SEQ ID NO:22)  
/MO2686, and MO2700 (SEQ ID NO:23) /MO2701 (SEQ ID NO:24)  
were used to PCR amplify the entire *lovE* open reading  
30 frame from plasmid MB2478. The PCR products differ in the  
amount of 5' and 3' vector sequence flanking the *lovE* open  
reading frame.

PCR was performed using a GeneMorph PCR mutagenesis  
kit (Stratagene, La Jolla, Ca) according to manufacturer's  
35 instructions to achieve medium and high range mutation  
frequencies.

Plasmid MB2478 was cut with *Asp*718/*Xba*I to release a  
1.7 kb fragment. The remaining 5.0 kb vector sequence  
completely lacks *lovE* ORF sequence.

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**Example 4: Transformation and Selection for G418R Isolates**

5 All PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. All vectors were gel purified using a QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions.

10 The mutagenesis strategy of Muhlrad *et al.* (*Yeast* 8:79-82 (1992)) was used which involves cotransforming a mutated PCR product and gapped plasmids into *S. cerevisiae*, and then screening for *in vivo* recombinants having the desired phenotype).

15 Transformation of *Saccharomyces cerevisiae* was accomplished by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol (Woods R.A. and Gietz R.D. *Methods Mol. Biol.* 177:85-97 (2001)) with a 1:5 molar ratio of vector:insert DNA to generate >55,000 *in vivo* recombinant transformants on SC-Ura plates. Transformants were transferred by replica printing to YPD plates containing 100 µg/ml G418 and allowed to grow for 2-4 days at 30°C (Figure 1).

20 Drug resistant clones were confirmed in secondary assays including growth on G418 concentrations up to 2000 µg/ml. The plasmid-dependence of the phenotype was determined by observing the re-appearance of drug sensitivity correlating with loss of the library plasmid. *lovE* variant plasmids were recovered from promising candidates (Hoffman and Winston (1986) *Gene* 57:267). More than 70 *lovE* variants were identified and definitively characterized by DNA sequence and/or restriction digestion analysis.

30 Table 3 summarizes the G418 resistance phenotype and sequence analysis of 26 of these variants.